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Unique Neuronal Tracers Show Migration and Differentiation of SVZ Progenitors in Organotypic Slices

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ABSTRACT: Continual neurogenesis in the subventricular zone (SVZ) of postnatal and adult mammalian forebrain has been well documented, but the mechanisms underlying cell migration and differentiation in this region are poorly understood. We have developed novel *in vivo* and *in vitro* methods to investigate these processes. Using stereotaxic injections of a variety of tracers/tracker [Cholera Toxin β subunit (CTb-), Fluorogold (FG), and Cell Tracker Green (CTG)], we could efficiently label SVZ cells. Over several days, labeled cells migrate along the rostral migratory stream (RMS) to their final differentiation site in the olfactory bulb (OB). The compatibility of these tracers/trackers with immunohistochemistry allows for cell labeling with multiple dyes (e.g., CTb and CTG) and/or specific cell antigens. To investigate the dynamics of migration we labeled SVZ progenitor cells with small injections of CTG and monitored the movements of individual cells in fresh parasagittal brain slices over several hours using timelapse confocal microscopy. Our observations suggest that tangential cell migration along the RMS occurs more rapidly than radial cell migration into the OB granule cell layer. To investigate migration over longer time periods, we developed an *in vitro* organotypic slice in which labeled SVZ progenitors migrate along the RMS and differentiate within the OB. The phenotypic characteristics of these cells *in vitro* were equivalent to those observed *in vivo*. Taken together, these methods provide useful tools investigating cell migration and differentiation in a preparation that maintains the anatomical organization of the RMS.

Keywords: subventricular zone; subependymal layer; olfactory bulb; cell migration; cell differentiation; organotypic culture

INTRODUCTION

The forebrain of both neonatal and adult rodents is characterized by persistent neurogenesis in the hippocampal dentate gyrus (Altman, 1965; Kaplan and Hinds, 1977; Kuhn et al., 1996) and the olfactory bulb (OB; Hinds, 1968a, 1968b; Altman, 1969; Bayer, 1983). Whereas the origin of the hippocampal dentate granule cells is still unclear, recent studies have shown that the newly generated neurons of the OB originate from a multipotent stem cell compartment located in the subventricular zone (SVZ; Luskin, 1993; Lois and Alvarez-Buylla, 1994; Weiss et al., 1996). These cells migrate tangentially along the rostral extension of the SVZ to the OB, following a highly defined pathway referred to as the "rostral migratory stream" (RMS). In adult animals, these undifferentiated migrating cells are characterized by expression of the immature, polysialylated isoform of the neural cell adhesion molecule (PSA-NCAM; Bonfanti and Theodosis, 1994; Rousselot et al., 1995). Migrating cells are organized in longitudinal chains, separated from the surrounding mature neuropil by a glial meshwork forming glial tubes (Doetsch and Alvarez-Buylla, 1996; Jankovski and Sotelo, 1996; Lois et al., 1996; Peretto et al., 1997). In neonatal rats, the SVZ is larger than in the adult, and a chain migratory arrangement within glial tubes is not detectable until approximately the third week postnatal (Peretto et al., 1999). Although the SVZ anatomical organization is quite different in newborn and adult animals, the main features of rostral migration are similar.

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A variety of studies suggest that cells from the SVZ continue to divide along the entire length of the rostral migratory pathway (Lois and Alvarez-Buylla, 1994; Luskin and Bone, 1994; Hauke et al., 1995; Menezes et al., 1995). Upon reaching the center of the OB, these cells migrate radially as single elements towards the granule and glomerular layer, where most cells assume the characteristic morphology of granule cells and juxtaglomerular interneurons respectively (Luskin, 1993; Lois and Alvarez-Buylla, 1994). However, it is unclear whether all the migrating cells give rise to neurons, and whether all these newly formed neurons survive and become incorporated into functional neuronal circuits. To address these issues a method for labeling large numbers of SVZ progenitor cells is needed.

In vitro, the adult SVZ generates neurospheres containing self-renewing, multipotential stem cells, whose differentiative fate can be controlled by single factors added to the culture. These factors can induce the generation of all three major cell types of the CNS: astrocytes, oligodendrocytes, and neurons (reviewed in Weiss et al., 1996). Experiments performed both *in vitro* and *in vivo* demonstrated that the septum, at the midline of the telencephalon and caudal to the SVZ, secretes the repulsive factor Slit guiding SVZ neuroblasts in their migration (Hu et al., 1996; Wu et al., 1999; Chen et al., 2001). Furthermore, an increasing number of specific molecules, including extracellular matrix, cell surface molecules, trophic factors, and tropic factor receptors are expressed selectively in the SVZ–RMS system (Bonfanti and Theodosis, 1994; Thomas et al., 1996; Perroteau et al., 1998; Conover et al., 2000). These results suggest that local molecular factors along the RMS and in the bulb may influence the migration and final commitment of these cells. However, despite numerous experimental studies performed *in vivo* and *in vitro*, the molecular mechanisms underlying the neurogenetic processes occurring in the SVZ and in the OB remain unclear.

The aim of the present study was to develop novel, easily applied methods for labeling cells in the SVZ thus making it possible to track their migration and differentiation as they migrate towards and differentiate in the olfactory bulb *in vivo* and *in vitro*.

METHODS

Stereotaxic Surgery and Tracer Injections

Stereotaxic injections were performed on CD1 strain postnatal day 1–2 mice (P1–P2; Charles River Labs; the day of birth was designated as P0) and adult CD1 strain mice (Charles River Labs). New-born mice were deeply anaesthetized by hypothermia, and their heads were immobilized on a custom neonatal stereotaxic apparatus maintained at 4°C during surgery. The skull was exposed by a skin incision and small holes drilled through the skull. Either 100–200 nL or 300–400 nL of each tracer was injected at stereotaxic coordinates of 0 mm bregma, 0.8 mm lateral to sagittal sinus, and 1.5 mm depth, by means of a glass micropipette and a pneumatic pressure injection apparatus (Picospritzer II, General Valve Corp, Fairfield, IL). After the micropipette was removed, the skin was sutured with 8.0 mm silk thread. The pups were quickly revitalized under a heat lamp and subsequently returned to the dam. The total duration under hypothermic anesthesia for postnatal mice was 15–20 min.

Adult animals were anesthetized by intraperitoneal injection of Nembutal (4–5 mg/100 mg body weight in 0.9% saline solution) and positioned in a stereotaxic apparatus (Stoelting, Wood Dale, IL). The mice were maintained on a warm platform at 35°C to maintain the body temperature constant during anesthesia. The surgical procedure followed the same steps described for neonatal mice, using stereotaxic coordinates of 0.5 mm anterior to bregma, 1 mm lateral to the sagittal sinus, and a depth of 2 mm. Following surgery, the animals were left under a heat lamp, and constantly monitored until recovery. All the experimental procedures were carried out in accordance with protocols approved by our institutional animal care and utilization committees.

Neuroanatomical Tracers

Several different neuroanatomical tracers were tested for their ability to label migrating cells in the rostral migratory stream. These tracers were, (1) cholera toxin β (CTb; 1% in 0.9% NaCl; List Biologicals, Campbell, CA, Catalog #104); (2) cholera toxin β FITC conjugate (CTb-FITC; 1% in 0.9% NaCl; Sigma Chemical Company, St. Louis, MO, Catalog #C1655); (3) 10 kDa biotinylated dextran lysine (BDA; 1% in 0.9% NaCl; Sigma Chemical Company, Catalog #B9139); (4) Fluorogold (FG; hydroxystilbamidine, methanesulfonate; 1% in 0.9% NaCl; Fluorochrome Incorporated, Englewood, CO); and (5) Cell Tracker Green CMFDA (10 mM in dimethylsulfoxide; Molecular Probes, Eugene, OR, Cat #2925). Individual tracers were injected into separate animals; however, in some experiments a mixture of 1% CTb and 1% BDA was injected. Double labeling with Cell Tracker Green and CTb were performed by sequential injection of 10 mM Cell Tracker Green and 1% CTb to the same stereotaxic coordinates.

Preparation of Forebrain Organotypic Slice Cultures

The brains from 24 h postinjection (dpi) mouse pups (P2–P4) were quickly removed under aseptic conditions, and placed into Petri dishes containing ice-cold dissection medium (Leibovitz's L-15, pH 7.4, Life Technologies/Gibco-BRL, Long Island, NY). Using a razor blade, the brains were cut in the coronal plane at the level of the inferior colliculus, and bisected along the mid-sagittal line. The two hemispheres were then placed with the medial portion face down into a Petri dish and embedded in a solution of 3% low gelling agarose (Sigma Chemical Corporation) in L-15 medium. A cutting block was obtained and transferred to a vibratome. The chamber of the vibratome was filled with cold dissection medium, and 300 μ m-thick parasagittal sections cut through the forebrain. These slices were moved carefully because any torsion, stretch, or compression trauma results in damage and subsequent widespread necrosis in those regions *in vitro*. Slices were collected into a Petri dish filled with the same medium. For each hemisphere, only one or two slices containing the SVZ and RMS could be recovered. One slice from each brain was immediately fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 1 h; the other slices were maintained *in vitro* as organotypic slices.

Organotypic slices were plated onto 30-mm Millicell inserts (Millipore, Bedford, MS) coated with poly-L-lysine and a thin layer of Matrigel (Becton-Dickson, Franklin Lakes, NJ). Sterile coverslips (22 x 22 mm) were prepared by washing in 100% ethanol, air drying, and autoclaving. The coverslips were then coated with 1 μ g/mL of poly-L-lysine (mol. wt. 70,000–150,000; Sigma Chemical) in Gey's balanced salts solution (GBSS; Life Technologies/GibcoBRL) for 1 h at 37°C in 5%CO₂, washed with GBSS, and coated with 100 L of Matrigel at 37°C in 5% CO₂ for 1 h. The inserts were placed into six-well dishes containing 2 mL of culture medium. This media was partially removed from the wells such that only a thin layer of liquid remained covering each slice. The organotypic slices were maintained in a humidified incubator (37°C, 5%CO₂, 23%O₂) for 1, 2, or 3 days. The slice culture medium consisted of Neurobasal medium (Life Technologies/GibcoBRL) supplemented with 1 N2 supplement (Life Technologies/GibcoBRL), 0.5 mM Glutamine (Life Technologies/GibcoBRL), and 25 μ g/mL Gentamycin (Life Technologies/GibcoBRL).

Tissue Processing

To analyze the pattern and the final fates of the cells stained with the different tracers/tracker, we examined the brains of newborn and adult animals at 1 to 7 days postinjection. The pups and the adult animals were deeply anesthetized with halothane inhalation or intraperitoneal injection of sodium pentobarbital (6.5 mg/100 g body weight), respectively. All the animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M PB (pH 7.4). All specimens were postfixed for a further 4 h, washed in 0.1 M PB for 15 min, and placed in 30% sucrose solution in 0.1 M PB for 24 h. The brains were embedded in Tissue-Tek optimal cutting compound

(OCT; Sakura Finetek, Torrance, CA), frozen on dry ice, and stored at -70°C until sectioned. Cryostat parasagittal sections (30 µm) were cut and collected as floating sections.

Organotypic slices were washed twice with 0.1 M phosphate-buffered saline (PBS), and fixed by immersion in 4% paraformaldehyde in 0.1 M PB for 1 h. The slices were then carefully processed and sectioned at 30 µm.

Visualization of Tracers

Cell Tracker Green and Fluorogold are intrinsically fluorescent, whereas CTb–FITC is directly conjugated with fluorescein. Antibodies to CTb were used to detect the unconjugated form of CTb following standard immunochemical techniques (see below). Biotinylated dextran was visualized by streptavidin conjugated with CY2, CY3, or CY5.

Immunohistochemistry

Immunohistochemical procedures were performed on sections derived either from *in vivo* or *in vitro* experiments. CTb was labeled using a goat polyclonal anti-CTb (1:5000; List Biological Laboratories, Catalog #703). PSA–NCAM was used as an endogenous marker of the migrating neuroblasts, and detected using a mouse IgM monoclonal antibody (1:1000; courtesy of Dr. Rougon, Marseille, France). Astrocytes were stained with a polyclonal rabbit antibody a-GFAP (1:5000; Boehringer-Mannheim, Indianapolis, IN, Catalog #814369).

Sections were rinsed twice in 0.1 M PBS, then incubated for 30 min in 1 M phosphate-buffered saline (PBS; pH 7.4) with 0.3% Triton X-100, 1% bovine serum albumin (BSA; Sigma Chemical Corporation), and 1% normal serum (from the species of origin of the appropriate secondary antibody). Sections were sequentially incubated with primary antibodies diluted in the above buffer for 16 h at 22°C, washed in PBS with 0.3% Triton X-100 (three washes of 5-min duration), and incubated for 1-h in the appropriate secondary antibodies conjugated with either CY2, CY3, or CY5 (1: 500; Jackson ImmunoResearch Laboratories, West Grove, PA). All incubations were performed at 22°C on a rotating platform. Some sections were counterstained by incubation in 5 nM Sytox Green Nucleic Acid Stain (Molecular Probes, Catalog #S7020) for 30 min at 22°C following immunohistochemical labeling.

Sections were then coverslipped with a DABCO (Sigma Chemical Corporation)-based antifade mounting media, and photographed on an Olympus FluoView confocal microscope fitted with standard excitation and emission filters for the visualization of CY2, CY3, and CY5.

Time-Lapse Confocal Microscopy

For time-lapsed studies animals were stereotactically injected with Cell Tracker Green or CTb–FITC. Sagittal slices (300 µm) of P2 mouse forebrain were obtained as described above. Selected slices containing the rostral migratory stream were immobilized between string grids and placed in a constant perfusion chamber in Minimum Essential Medium (Life Technologies/GibcoBRL) supplemented with 5% Fetal Bovine Serum (FBS; Life Technologies/Gibco-BRL), 0.5 mM glutamine (Sigma Chemical Corporation), and 25 µg/mL gentamycin (Life Technologies/GibcoBRL). The perfusion chamber was fixed to the stage of an Olympus FluoView confocal microscope and maintained at 37°C by means of two thermostatically driven heater elements (Cell Microcontrols, Norfolk, VA). The slice was constantly perfused with 0.5 mL/min steady flow of oxygenated culture medium. Each slice was maintained for 1 h in the chamber prior to imaging, and then imaged for up to 4 h with cells photographed every 15 min under a 40x water immersion objective. In several cases, labeled cells migrated out of the field of view and the observation field was shifted to follow the cell. Also at the same time the focal plane of the cell was constantly monitored and changed as required. All migrating cells were 40–60 µm from the upper surface of the slice.

Photographs

Digital microscopy images were captured by means of confocal acquisition (FluoView personal confocal microscope, Olympus Instruments, CA), or a Leica DMRX microscope fitted with a 35-mm camera (for photographing fluorogold). The digital images were brightness, color, and contrast balanced, assembled into montages using Corel-Draw 10 (Corel Corporation, USA), and printed on a Fuji Pictography 3000 printer (Fuji PhotoFilm Co., Tokyo, Japan). No additional digital image manipulation was performed.

RESULTS

Labeling of SVZ Migrating Cells in Adult and Postnatal Mice

Previous methods used to label SVZ cells involve injections of either DiI or retroviral particles (Luskin 1993; Lois and Alvarez-Buylla, 1994). To identify additional markers to label these cells we tested the neural tracers, Fluorogold (FG), subunit β of cholera toxin (CTb) and biotinylated dextran (BDA). These three tracers are commonly used in neuroanatomy for studying neuronal connections. We also tested Cell Tracker Green CMFDA (CTG), which has been reported to be a vital probe for long-term tracing of living cells *in vitro* (Molecular Probes) but has not been previously used as an injected tracer. Each tracer was injected in the forebrain of newborn and adult mice at the stereotaxic coordinates corresponding to the anterior part of the SVZ lining the lateral ventricles. The labeling efficiency of each tracer for cells that migrate into the bulb was assessed in neonatal and adult mice following survival times of 1 to 7 days.

Cell Labeling with CTb and Cell Tracker Green.

SVZ injections with either CTb or CTG yielded a large number of cells selectively distributed along the RMS at short survival times [Fig. 1(a)] and in various cell layers of the olfactory bulb at longer survival times [Figs. 1(d)–(g) and 2]. Increasing the volume of tracer injected increased the number of labeled cells, but did not significantly increase the labeling intensity of individual cells. At a cellular level, both markers stained the entire cell, yielding a Golgi-like appearance of the soma and processes in migrating cells. The leading and trailing processes including the neuronal growth cone were clearly visible [Fig. 1(c)]. CTG homogeneously filled the soma and processes of labeled cells [Fig. 1(d)], but the thin processes of differentiated cells photobleached rapidly when observed under fluorescent light. CTb filled the soma and fine processes of labeled cell, but was often more punctate in appearance than CTG [Fig. 1(c)–(f)]. Compared to the direct visualization of the CTb–FITC conjugate (not shown), the immunohistochemical amplification of CTb resulted in more robust staining of fine processes.

In adult animals, double immunostaining for Ct b and PSA–NCAM showed that 2 days after injection most migrating CTb-labeled cells in the rostral migratory stream [Fig. 1(a)–(b)] and subventricular region of the bulb [Fig. 1(d)–(f)] expressed PSA–NCAM. Within the RMS, CTb-immunopositive cells were organized as chains of labeled cells or as isolated (or single) cells, although these latter cells could be part of chains, containing unlabeled cells. Labeled cells in the RMS had morphology typical of migrating neuroblasts, with an elongated cell body, a trailing process and a leading process terminating with a growth cone oriented in the direction of migration [Fig. 1(c)]. Seven days after injection with either CTb or CTG a large number of radially oriented cells were found in the granular layer of the MOB [Fig. 1(d)], although there were still undifferentiated migrating cells present in the RMS extension in the MOB. The number and distribution of cells labeled by CTb or CTG injection was similar; however, potentially these tracers/trackers could be preferentially labeling different populations of migrating cells. To address this issue, sequential injections of CTb and CTG to the same stereotaxic coordinates demonstrated that cell migrating from the SVZ could

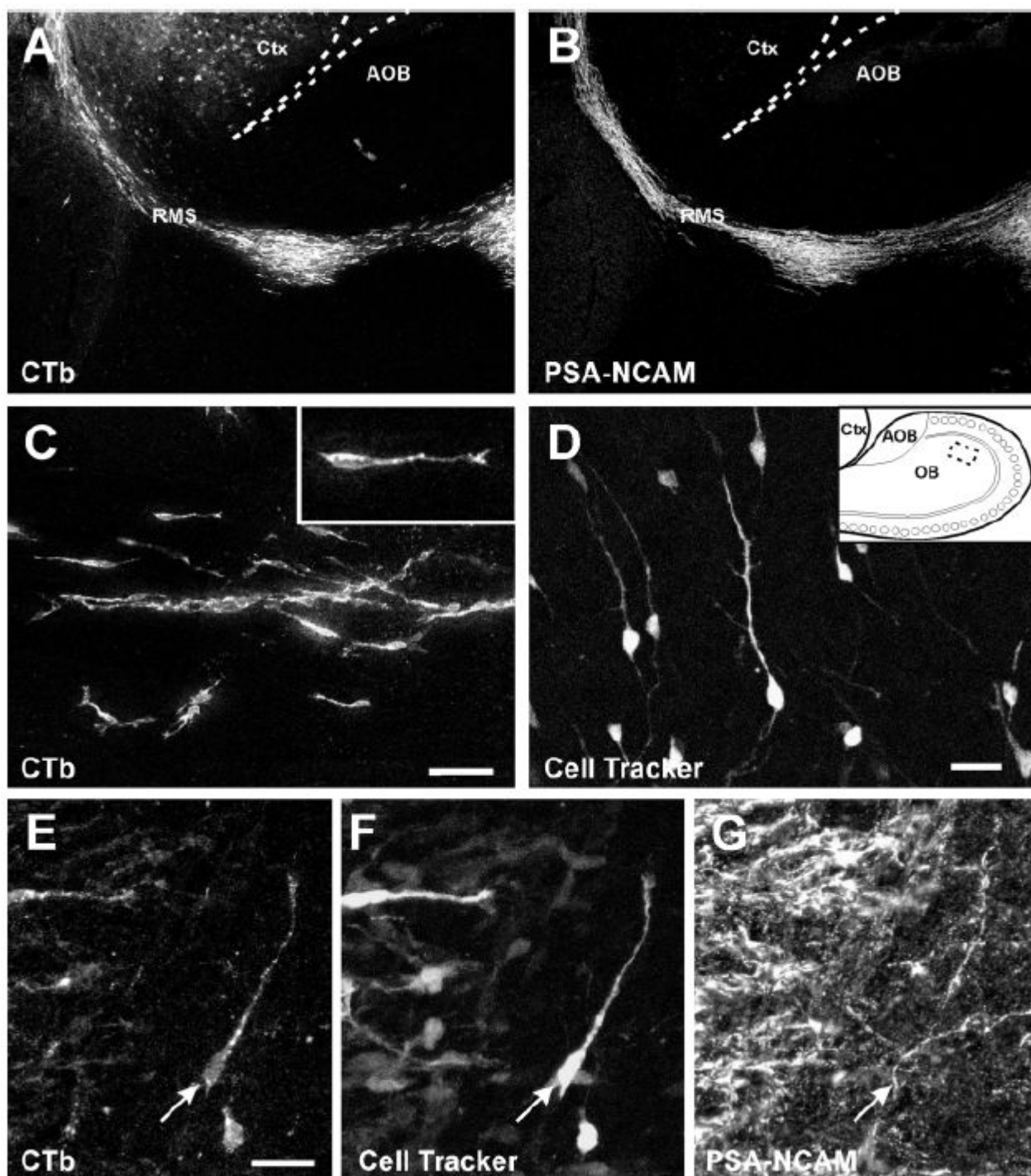


Figure 1 Photomicrographs of CTb, Cell Tracker Green, and PSA-NCAM-labeled cells in parasagittal sections of the adult mouse forebrain. (A,B) Two days following CTb injection, numerous CTb cells are present along the rostral migratory stream (RMS). Double immunostaining with CTb and PSA-NCAM indicated that most of the PSA-NCAM immunopositive migrating cells (B) were also labeled for CTb (A). (C) At higher magnification, CTb labeled cells were organized in chains or as single elements showing an elongated cell body with a short trailing process and a longer leading process and growth cone (insert panel). (D) Seven days following tracer/tracker injection radially oriented cells were present throughout the granule cell layer of the bulb. The morphology of these cells was identical to granule cells, with radial orientation and numerous small protrusions, spines, from the apical process. The insert diagram indicates the area of olfactory bulb shown in the panel. (E,F,G) A neuroblast migrating from the SVZ in the outer layers of the OB triple labeled with CTb (E), Cell Tracker Green (F), and PSA-NCAM (G). Accessory olfactory bulb (AOB), frontal cortex (Ctx). The scale bar represents 200 μ m in (A) and (B), 50 μ m in (C) (20 μ m in the insert panel), and 20 μ m in (D)–(G).

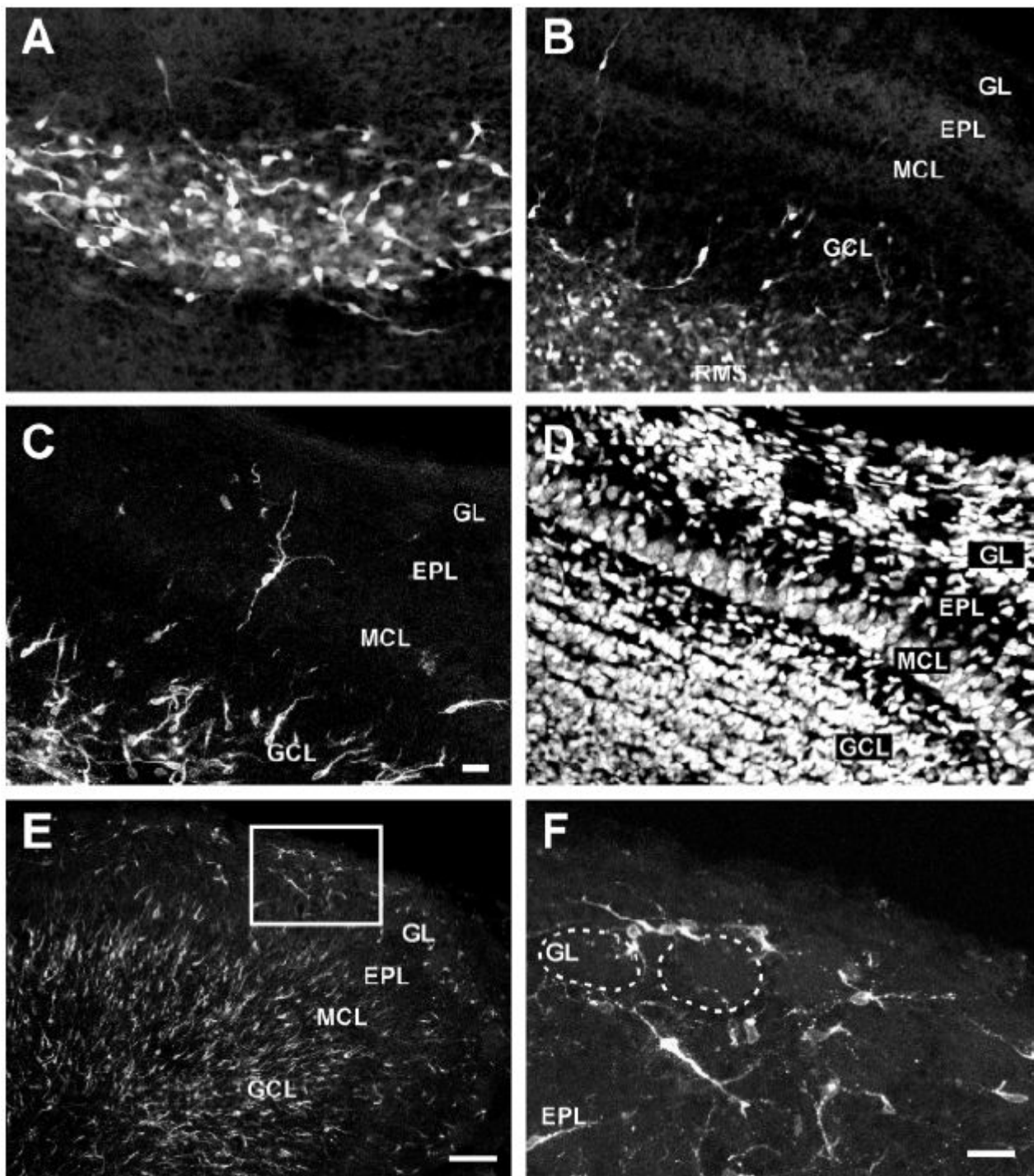
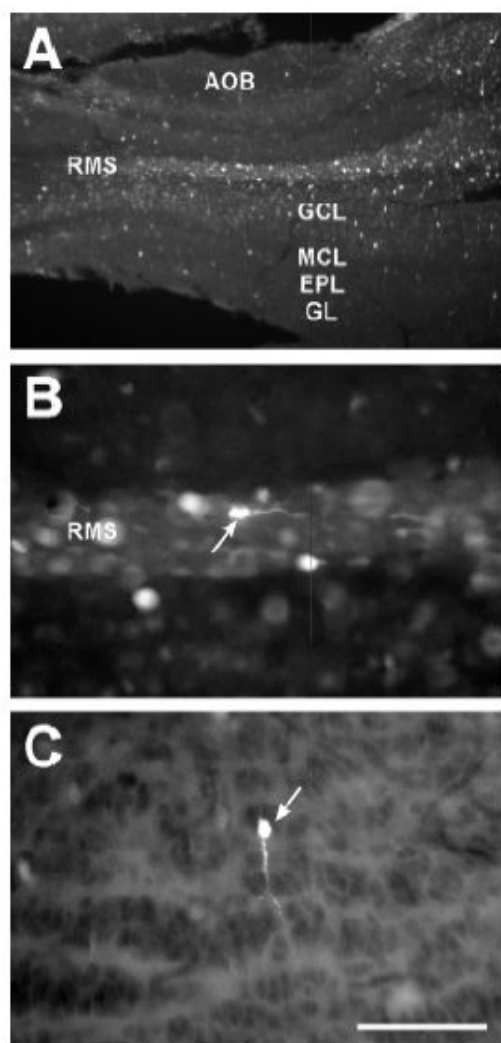


Figure 2 Parasagittal sections of the forebrain of newborn mice 1–2 (A,B), 3–4 (C,D), and 6 (E,F) days postinjection. (A) Numerous CTb labeled cells migrating in the rostral migratory stream 1–2 days post injection. (B) In the olfactory bulb of a 1–2-daypost injection animal, only a few scattered cells have left the RMS and entered the granule cell layer. (C) At 3–4 days postinjection labeled cells are present throughout the granule cell layer and scattered cells are present in the external plexiform and glomerular layers. (D) Nuclear counterstaining of the same section in (C) showing the position of the bulb layers. (E) Six days postinjection; labeled cells are distributed widely throughout the bulb. (F) Higher magnification of the insert in (E) showing dye-labeled juxtglomerular neurons. Several glomeruli are indicated by dashed outlines. Rostral migratory stream (RMS), granule cell layer (GCL), mitral cell layer (MCL), external plexiform layer (EPL), glomerular layer (GL). Scale bar = 20 μ m in (A), 50 μ m in (B), 20 μ m in (C) and (D), 100 μ m in (E) and 20 μ m in (F).

be labeled by multiple dyes [Fig. 1(e)–(f)]. However, although most labeled cells contained both the tracers injected, some cells labeled with only CTb and others with only CTG. This probably reflects slight variability in the injection sites and in the ability of the two dyes to spread through the tissue. The identity of these double-labeled cells as migrating neuroblasts was confirmed by triple labeling with Cell Tracker Green, CTb, and PSA–NCAM [Fig. 1(e)–(g)].

In newborn mice 24 h postinjection, numerous CTb- and CTG-labeled cells were observed along the length of the RMS to the caudal OB. One to 2 days postinjection, most of the labeled cells were still restricted to the SVZ in the center of the bulb; only a few scattered cells with radial orientation were present within the granular layer of the MOB [Fig. 2(a) and (b)]. Three to 4 days postinjection the number of stained cells in the granular layer increased dramatically. Both undifferentiated cells with a migratory morphology, and differentiated granule celllike interneurons were labeled [Fig. 2(c)]. Scattered cells were also observed in the external plexiform and glomerular layers (EPL and GL) extending processes into or between glomeruli. Some of these cells displayed complex process arbors and morphologically resembled juxtglomerular interneurons. At 6 days postinjection, numerous morphologically differentiated cells were found throughout all the different layers of the olfactory bulb [Fig. 2(e)–(f)]. Most of these cells were located within the granule cell layer. Many cells were also present in the glomerular layer [Fig. 2(f)]. Only scattered cells were present in the external plexiform layer. The development of these labeled cells into neurons with complex dendritic morphology indicates that neither dye interferes with the differentiation of the cells. Moreover, there was no evidence for neural degeneration due to cytotoxicity at the injection site using either CTb or CTG.



Cell Labeling with Fluorogold. Fluorogold is another commonly used neuroanatomical tracer. We injected fluorogold into the subventricular zone of both newborn and adult mice. Six days following Fluorogold injection in either postnatal or adult mice, labeled cells were present along the RMS and throughout the layers of the olfactory bulb. Both undifferentiated migratory cells and morphologically differentiated neurons were observed [Fig. 3(a)–(c)]. Fluorogold labeling was only weakly visible in the fine processes of differentiated cells and rapidly photobleached from these processes. All the animals injected with fluorogold showed an area of necrotic tissue near the injection site, which is often observed when fluorogold is used as a neuroanatomical tracer.

Cell Labeling with BDA. Injections of BDA into the subventricular zone of either postnatal or adult animals never labeled cells that migrate in the RMS or olfactory bulb. However, cells near the injection site and in the underlying cortical regions were labeled by BDA.

Figure 3 Photomicrographs of fluorogold-labeled cells in the adult forebrain 6 days after stereotaxic injection of the tracer. (A) Low-magnification sagittal olfactory bulb section showing the presence of stained cells in the rostral migratory stream (RMS) and bulb. (B) Fluorogold-labeled cells are tangentially oriented in the RMS and have a distinct leading process (arrow). (C) In the olfactory bulb granule cell layer (GCL), differentiated fluorogold-labeled cells have the morphological appearance of granule cells with a small soma and long apical dendrite

(arrow). Accessory olfactory bulb (AOB), mitral cell layer (MCL), external plexiform layer (EPL), glomerular layer (GL). Scale bar = 200 μ m in (A), and 50 μ m in (B) and (C).

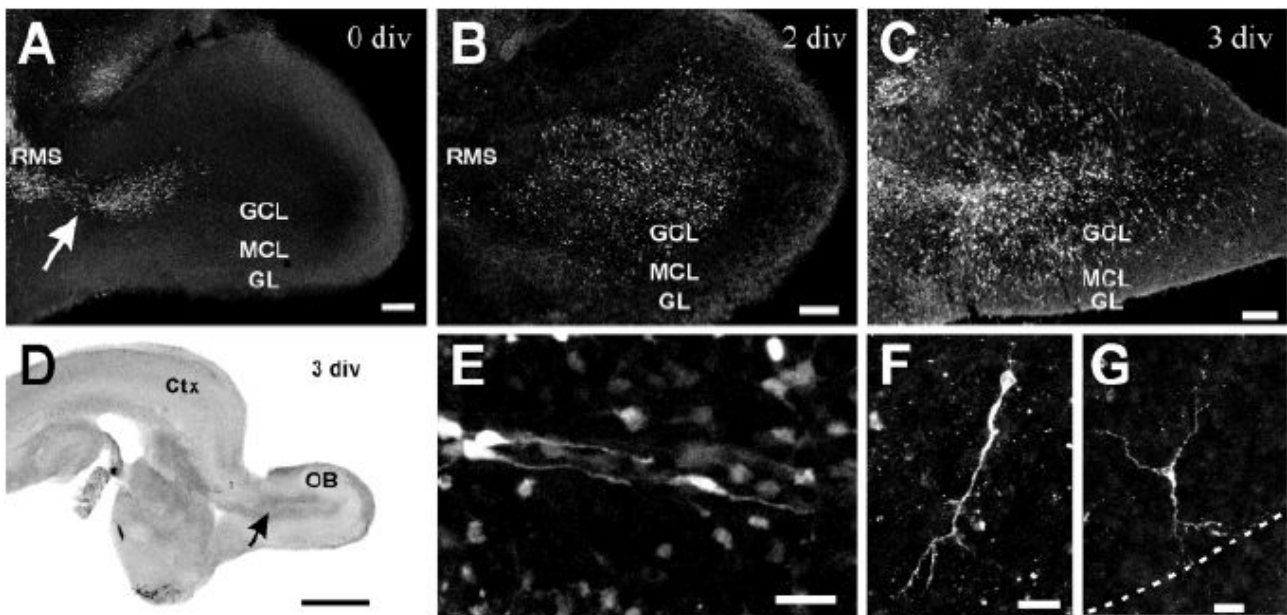


Figure 4 Photomicrographs showing low (A–C) and high (E–G) power view of OB parasagittal sections of newborn mouse after zero (A) two (B,E) and three (C,F,G) days *in vitro* (div). CTb stereotaxic injections were performed 1 day before starting the culture protocols. (A) One day postinjection, corresponding to 0 div, labeled cells are localized in the RMS in the caudal part of the OB (arrow). (B) After 2 days *in vitro*, CTb-labeled cells are present throughout the GCL. (C) After 3 days *in vitro*, labeled cells are diffusely distributed throughout the OB. (D) Postnatal parasagittal forebrain slice cultures after 3 days *in vitro* (3 div). These organotypic slices retain normal tissue architecture and anatomical organization of the SVZ migratory pathway (arrow). (E) Higher magnification of the same section as (B) at the level of the RMS at 2 days *in vitro* reveal the occurrence of cells with an elongated cell body and leading process characteristic of migrating cells (E). (F) A differentiated cell in the granule cell layer after 3 days *in vitro*. Cells located in this layer are morphologically similar to granule cells, with a long apical dendrite protruding through the external layers of the OB before branching. (G) In the glomerular layer at the edge of the slice the morphology of cells is more variable with wide dendrite arbor. Rostral migratory stream (RMS), granule cell layer (GCL), mitral cell layer (MCL), external plexiform layer (EPL), glomerular layer (GL), olfactory bulb (OB), cortex (Ctx). Scale bar = 200 μm in (A), 100 μm in (B) and (C), 1 mm in (D), and 20 μm in (E)–(G).

To confirm that the injections were correctly positioned to label cells in the SVZ, we injected a mixture of BDA and CTb. Four days after the BDA and CTb mixture was injected into the SVZ, CTb-labeled cells were abundantly present. These CTb labeled cells were distributed along the RMS and throughout the MOB, whereas BDA labeling was completely absent from these regions. The BDA labeling was restricted to a population of morphologically differentiated cells localized in the underlying striatum and in a few cells lining the rostral border of the lateral ventricle (data not shown).

Cell Migration and Differentiation in the SVZ–RMS System Persists in Organotypic Slice Cultures

SVZ cell migration and differentiation have been studied in dissociated (reviewed in Weiss et al., 1996) and explant cultures (Hu and Rutishauser, 1996; Wichterle et al., 1997). A potential limitation of these models is that they disrupt the anatomical organization of the pathway and the normal tissue architecture. In an attempt to overcome this limitation, we developed an organotypic slice culture that maintains the anatomical organization of the pathway within the 300- μm sagittal slice. Prelabeling these SVZ-derived cells with CTb and/or CTG demonstrated that migration of cells occurred in the slices. Organotypic slices were maintained *in vitro* for up to 3 days (3 div) on a thin Matrigel layer (Fig. 4). The general architecture of the tissue was well preserved over several days; however, in some slices (~20% of slices) large round cells morphologically similar to macrophages were observed in the slice. The presence of these macrophage-like cells was associated with ge-

neral fragility in the slice and loss of cell birefringence under highmagnification phase contrast or DIC optics indicated widespread damage in these slices. Immunohistochemical analyses performed after 3 div using antibodies directed against PSA–NCAM and the astrocytic marker GFAP, showed distribution patterns for these markers that were similar to those found *in vivo* at comparable ages (data not shown). These observations suggested that the general neural and glial arrangements in the organotypic slices were maintained during the culture period. Culture periods longer than 3 days were not systematically examined, but many researchers report anecdotal evidence that static organotypic cultures of greater than 1 week *in vitro* exhibit numerous areas of cellular degeneration.

Organotypic slices fixed immediately after sectioning (1 day postinjection; corresponding to 0 div) contained labeled cells that had already reached the caudal OB; however, the majority of cells were still distributed along the entire RMS [Fig. 4(a)]. At 2 div the labeled cells had spread from the RMS into the center of the bulb and throughout the granule cell layers. Most of these cells exhibited the characteristic morphology of migrating neurons [Fig. 4(e)], similar to that seen *in vivo* 2–3 days postinjection. Thus, labeled cells continue to migrate from the RMS into the olfactory bulb, *in vitro*. At 3 div, the migrating cells were distributed throughout all layers of the bulb including the glomerular region. Most of the labeled cells had differentiated into neurons with a granule cell-like appearance [Fig. 4(f)] and juxtglomerular cell-like neurons in the outer bulb [Fig. 4(g)]. However, in a deafferented slice there are no olfactory receptor neuron axons forming glomeruli. Labeled cells in the glomerular layer had multiple dendrites, compared to the single dendrites present on cells in the granule cell layer. Many of labeled juxtglomerular cells have one or more of the dendrites forming a tuft of secondary processes at the distal end of the primary dendrite [Fig. 4(g)].

Migrating Cells Imaged with Time-Lapse Confocal Microscopy in Organotypic Slices

To date, studies of cells migrating along the RMS have relied upon observations of fixed tissue at different times (Lois and Alvarez-Buylla, 1994; Luskin and Boone, 1994) or explants of SVZ in which cells migrate into a matrigel matrix (Wichterle et al., 1997). By using a combination of CTb–FITC or CTG labeling with confocal microscopy we directly observed the migration of cells along the RMS. Individual cells approximately 40–60 μm from the surface of the slice were imaged every 15 min for several hours. A critical factor in these experiments was photobleaching and phototoxicity of the dye from the intense laser light used by the confocal. Preliminary experiments indicated that CTb–FITC fades rapidly, and exhibited significant phototoxicity to the labeled cells. However, CTG exhibited a high resistance to photobleaching and cells labeled with CTG manifested no signs of phototoxicity during imaging. Thus, CTG was used preferentially for time-lapse observations.

Migrating neurons were observed to extend a leading process followed by translocation of the soma in the direction of the leading process (Figure 5). The six cells observed within the RMS of different slices migrated at different rates (60–150 $\mu\text{m}/\text{h}$, with an average rate of 105 $\mu\text{m}/\text{h}$), and exhibited “saltatory” movement: i.e., periodic movement at up to 180 $\mu\text{m}/\text{h}$ followed by periods of slower migration, similar to that observed for dissociated SVZ cell migration (Wichterle et al., 1997). Several cells that migrated radially from the RMS into the bulb were monitored, and all of these cells moved at slower rates (20–35 $\mu\text{m}/\text{h}$) than tangentially migrating cells in the RMS. This finding is consistent with different migration rates observed in dissociated models for cells migrating in chains or *de novo* (Wichterle et al., 1997). The preliminary work on time-lapse migration in these slices shows that organotypic slices of the forebrain can be successfully used to directly observe cell migration.

DISCUSSION

Long-distance tangential cell migration in the forebrain of newborn and adult rodents has been previously demonstrated by means of stereotaxic injections of retroviral and lipophilic dye markers. Luskin (1993), using retroviral lineage tracing, demonstrated that in postnatal animals, progenitor

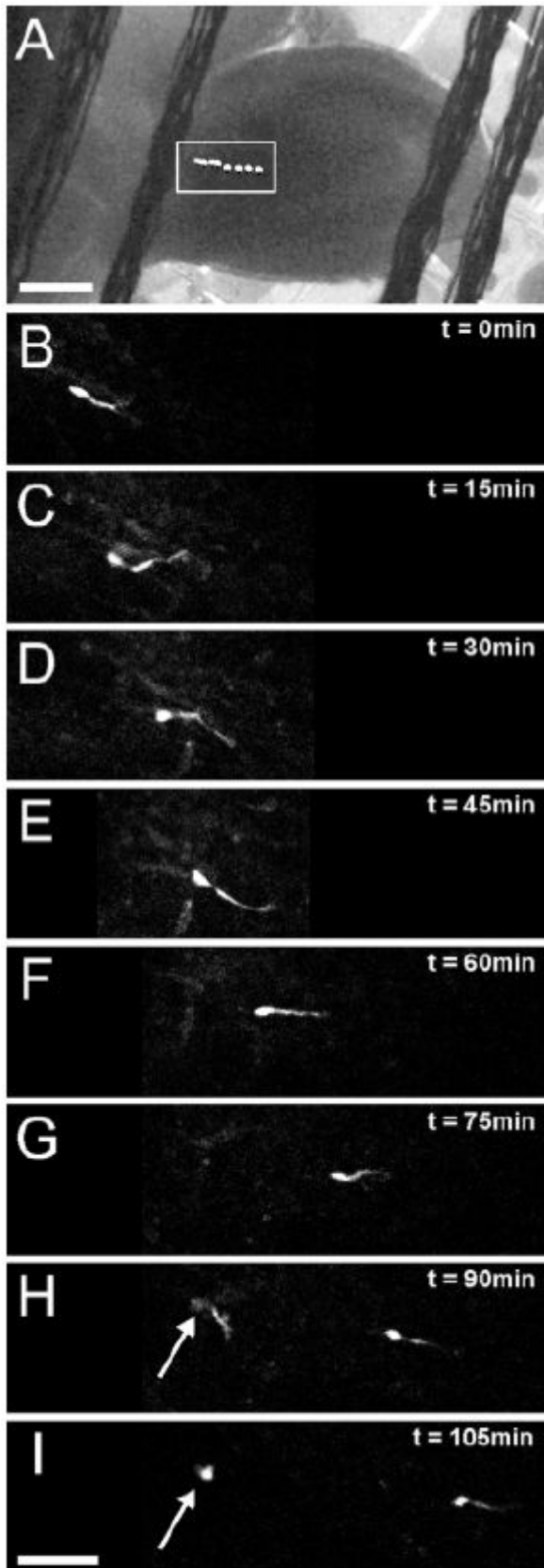


Figure 5 Time-lapse confocal microscope imaging of a Cell Tracker Green-labeled cell migrating tangentially along the RMS in an organotypic slice of a newborn mouse forebrain. (A) shows a low magnification of the slice, immobilized between the grids of the cell perfusion chamber. The box in (A) is a schematic overlay of the position of the imaged cell corresponding to the following frames (B)–(I). (B)–(I) This cell was monitored with time-lapse fluorescence confocal microscopy for 105 min. The time corresponding to each frame (B)–(I) is noted in the upper right corner. The frames in (F)–(I) were shifted 25 μm vertically to keep the cell centered in that axis of the figure. The arrow indicates a second labeled cell migrating into the plane of focus during the last two frames [(H) and (I)]. The scale bar = 400 μm in (A) and 50 μm in (B)–(I).

cells from a discrete region of the SVZ migrate into the olfactory bulb. These cells enter the olfactory bulb and differentiate into granule and juxtaglomerular neurons. Similar results were obtained on adult mice by means of stereotaxic microinjections of ^3H Thymidine, BrdU and DiI (Lois and Alvarez-Buylla, 1994.). However, many questions about the proliferation, migration, and final commitment of the progenitor cells of the SVZ remain unanswered. For example, it is not known if the migrating neuroblasts are a homogeneous population of precursor cells that differentiate into different types of interneurons upon reaching the OB, or if they are composed of different populations of cells precommitted to specific fates. It is also not known if all the newly formed migrating cells survive and what proportion is incorporated into functional neuronal circuits. To begin to address these issues we developed simple methods for labeling large numbers of SVZ progenitor cells that are compatible with immunohistochemical procedures.

In this study, we assessed the effectiveness of several neuroanatomical tracers, fluorogold (FG), subunit β of cholera toxin (CTb), and Cell Tracker Green CMFDA (CTG) to label cells migrating from the SVZ. The newly generated cells of the SVZ of both newborn and adult mice were effectively labeled by stereotaxic injections of CTb, CTG, and FG. These SVZ cells migrated into the olfactory bulb where they differentiated into a variety of different cell types. These tracers/trackers offer several advantages over retroviral particles and DiI labeling methods. Retroviral and DiI injection methods used previously (Luskin, 1993; Lois and Alvarez-Buylla, 1994) label only small numbers of cells. By contrast, we routinely labeled large numbers of migrating cells. Reducing the volume of the reagent injected results in fewer cells labeled so that individual cells can be easily resolved along the RMS for time-lapse microscopy. Thus, the versatility of CTb, FG, and CTG make them useful tools investigating the migration and the differentiation of large populations of SVZ cells. FG labels migratory cells from the SVZ, but provides only faint staining in the fine processes of differentiated cells. This, taken with the cytotoxicity observed at the injection site with FG, make it less attractive than CTb or CTG for long-term studies.

The use of tracers/trackers in studying final cell determination in the bulb is facilitated by the compatibility of these compounds with immunohistochemistry, in contrast to the difficulty of combining this technique with lipophilic DiI labeling. Also, the wide range of tracers/trackers available from commercial sources (e.g., CTb, CTb– FITC, CTb– TRITC, CTb– Biotin, Cell Tracker, etc.) allow for injection and visualization of multiple tracers used simultaneously at different locations and/or times.

Among the tracers tested, only BDA failed to label any migrating neuroblasts. Injections consisting of a mixture of CTb and BDA, resulted in only CTb labeled cells in the RMS and olfactory bulb. This finding demonstrates that the lack of BDA labeling is not due to any positional problem related to the injection site in BDA injections. These experiments used a 10-kDa biotinylated dextran lysine tracer. Although we consider it unlikely, it is possible that other molecular weight dextrans may label progenitor cells in the SVZ.

The SVZ Gives Rise to Different Cell Types

SVZ progenitor cells labeled with CTb and CTG migrated to the olfactory bulb and differentiated primarily into granule cells and juxtaglomerular cells. However, a small number of labeled cells were also present in the external plexiform layer (EPL) of the olfactory bulb. Luskin (1993) described SVZ-derived cells in the EPL comprising only 1– 10% of the final placement of the migrating cells into the OB. Moreover, Bonfanti and Theodosis (1994) described the presence of isolated PSA– NCAM immunopositive cells, with a bipolar morphology, in the EPL of the adult rat OB. The EPL cells stained with CTb and CTG show a branched morphology characteristic of differentiated cells, but do not have the morphology of tufted cells, and could represent a class of short axon or Blanes cells. The fate of SVZ-derived cells in the bulb has mostly been determined by morphology and soma location. In the glomerular layer there are several different classes of neurons (dopaminergic, CCK containing, etc.), and the developmental origin of each class has yet to be determined. A more detailed study, both on adult and postnatal animals with other immunohistochemical neuro-

transmitter markers, will be needed to better characterize all the different cell types that derive from the SVZ in both newborn and adult rodents.

Cell Migration and Differentiation in Organotypic Slice Cultures

In recent years, several *in vitro* experimental models have been applied to the study of cell neurogenesis and migration from the SVZ. The two commonly used models are dissociated cell cultures and tissue explants. The dissociated cell model consists of cells isolated from the SVZ and maintained *in vitro*. When these cells are stimulated with polypeptide growth factors, such as epidermal growth factor (EGF) and basic fibroblast growth factor (b-FGF), they form microspheres containing both undifferentiated multipotential stem cells and differentiated cells of neuronal and glial lineage (reviewed in Weiss et al., 1996; Weiss and van der Kooy, 1998). This *in vitro* approach has been extensively used in studies addressing the specific characterization and discrete localization of neural stem cells in the SVZ of adult brain (reviewed in Morshead and van der Kooy, 2001). *In vitro* studies using tissue explants obtained from the SVZ of newborn brain have been mainly focused on the mechanisms of cell migration. These studies provided direct evidences for the presence of a repulsive septum-derived factor, Slit, able to guide the migrating cells (Hu and Rutishauser, 1996; Wu et al., 1999; Chen et al., 2001). In this model homotypic, also termed chain migration, and *de novo* neuronal migration of SVZ neuroblasts was demonstrated (Wichterle et al., 1997). Although cell culture and tissue explants are important tools for studying the mechanisms of cell migration and differentiation, both these models involve significant alterations to the normal anatomical and functional relationships of the cellular elements in the SVZ, RMS, and OB. An attractive alternative to either dissociated cells or explants are organotypic slice cultures. Indeed, organotypic slice cultures obtained from other brain regions show that neurons differentiate and develop cytoarchitectural organization similar to that observed *in vivo* (reviewed in Gahwiler et al., 1997).

We developed an organotypic slice culture of neonatal mouse forebrain that preserves the three-dimensional cytoarchitecture of the SVZ, RMS, and OB. This organotypic slice can be maintained *in vitro* for several days with healthy appearance and cytoarchitecture similar to the comparable *in vivo* stage. Static cultures, similar to those used in this study, are generally considered to maintain the health of the tissue for approximately 1 week *in vitro*. To maintain organotypic slices for longer periods of time roller tube systems are often employed (Gahwiler, 1981). Cell migration and differentiation in the olfactory system only requires several days; thus, long-term roller environment cultures are not necessary in this case. SVZ cells labeled with CTb or CTG demonstrated that migration into the olfactory bulb was taking place *in vitro*. Many of the cells migrating into the olfactory bulb differentiate into cells with the morphological appearance of granule and periglomerular interneurons. The lack of olfactory input in the slices does not appear to perturb the migration and differentiation of the newly generated cells over several days *in vitro* compared to the equivalent *in vivo* stage. The lack of ORN input to the slices is similar to *in vivo* sensory deprivation models. Brunjes and colleagues found that long-term unilateral nares occlusion, a model of sensory deprivation, results in a smaller olfactory bulb (Brunjes and Borror, 1983; Brunjes, 1985; Cummings et al., 1997). In nares occluded animals, the rate of cell proliferation and migration was unaffected, and the reduction in bulb size was due to increased cell death (Frazier-Cierpial and Brunjes, 1989). We hypothesize, that if organotypic olfactory bulb slices are maintained for long periods *in vitro* cells will migrate into the bulb normally, but may undergo enhanced cell death in the absence of sensory input. The use of these short-term organotypic slices provides a potentially useful tool for investigating the molecular mechanisms that regulate the migration and differentiation of cells into the olfactory bulb in early development.

Direct Observation of Cell Migration along the RMS in Organotypic Slice Preparations by Time-Lapse Confocal Microscopy

Time-lapse microscopy of living cells has been used for many years to study cell migration and process elongation. More recently, time-lapse digital confocal imaging of cell migration in living brain slices has shown how these cells migrate through an intact cytoarchitecture (Kakita and Goldman, 1999). In our organotypic slices of mouse forebrain, we were able to monitor the migration of cells labeled with CTG for several hours. The only limits to observing these cells is photobleaching of the dye, phototoxicity, and the inability of a single photon confocal to image cells deeper than $\sim 80\ \mu\text{m}$ into the slice. Within these limitations, cells in the RMS and bulb can be imaged over hours and potentially days in organotypic cultures.

The migration rate of SVZ-derived cells in organotypic slices is higher than those previously estimated on fixed tissue at different times (Luskin and Boone, 1994; Lois and Alvarez-Buylla 1994). However, Wichterle et al. (1997) reported migration rates for cells derived from SVZ explants that are consistent with those observed in the organotypic slices. SVZ-derived cells migrating from the explant are organized in chains and migrate at $\sim 122\ \mu\text{m}/\text{h}$ and cells that are not organized in chains migrate at only $\sim 33\ \mu\text{m}/\text{h}$. These rates are similar to those observed in the organotypic slices for CTG-labeled cells in the RMS and radial migration in the OB, respectively. These findings indicate that organotypic slices can be a useful tool for examining both chain migration in the RMS and *de novo* radial migration into the OB layers.

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